Published in "The EMBO Journal 26(24): 5109–5119, 2007"
which should be cited to refer to this work.

An acetylation/deacetylation cycle controls the export of sterols and steroids from *S. cerevisiae*

Rashi Tiwari, René Köffel[1] and Roger Schneiter*

Department of Medicine, Division of Biochemistry, Institute of Biochemistry, University of Fribourg, Fribourg, Switzerland

Sterol homeostasis in eukaryotic cells relies on the reciprocal interconversion of free sterols and steryl esters. Here we report the identification of a novel reversible sterol modification in yeast, the sterol acetylation/deacetylation cycle. Sterol acetylation requires the acetyltransferase *ATF2*, whereas deacetylation requires *SAY1*, a membrane-anchored deacetylase with a putative active site in the ER lumen. Lack of *SAY1* results in the secretion of acetylated sterols into the culture medium, indicating that the substrate specificity of *SAY1* determines whether acetylated sterols are secreted from the cells or whether they are deacetylated and retained. Consistent with this proposition, we find that acetylation and export of the steroid hormone precursor pregnenolone depends on its acetylation by *ATF2*, but is independent of *SAY1*-mediated deacetylation. Cells lacking Say1 or Atf2 are sensitive against the plant-derived allylbenzene eugenol and both Say1 and Atf2 affect pregnenolone toxicity, indicating that lipid acetylation acts as a detoxification pathway. The fact that homologues of *SAY1* are present in the mammalian genome and functionally substitute for *SAY1* in yeast indicates that part of this pathway has been evolutionarily conserved.

Keywords: cholesteryl acetate; detoxification; secretory pathway; sterol esterase; yeast

Introduction

Sterols are essential lipids of most eukaryotic cells and serve both important structural and signaling functions. Sterols are synthesized by enzymes located in the ER membrane and are enriched in the plasma membrane where they increase the permeability barrier of the membrane and thus are important to maintain the membrane potential (Haines, 2001). Steryl esters, on the other hand, serve to store metabolic energy in form of fatty acids and are deposited in intracellular lipid droplets. The synthesis and hydrolysis of steryl esters is important to maintain sterol homeostasis as the steryl ester pool conceptually serves to buffer both excess and lack of free sterols (Chang *et al*, 2006).

Sterols are generally regarded as being stable and long-lived lipids. In metazoans, they are converted to steroids, ecdysone, vitamin D, oxysterols, and bile acids, and serve as lipid anchor for signaling proteins of the hedgehog family (Russell, 2000, 2003; Chang *et al*, 2006). However, sterol-like molecules can also have adverse effects and act cytotoxic, interfere with endocrine signaling through nuclear hormone receptors, and even elicit epigenetic transgenerational phenotypes (Anway *et al*, 2005).

Yeast contains two ER-localized acyl-CoA:sterol acyltransferases, Are1 and Are2, that catalyze the synthesis of steryl esters. Mutants lacking steryl esters, however, are viable, indicating that steryl ester synthesis is not essential under standard growth conditions (Yang *et al*, 1996; Yu *et al*, 1996). The hydrolysis of steryl esters, on the other hand, is catalyzed by a family of three membrane-anchored lipases, Yeh1, Yeh2, and Tgl1. Triple mutants lacking steryl ester hydrolase activity are again viable under standard growth conditions, indicating that similar to their synthesis, hydrolysis of steryl ester is dispensable for growth under standard conditions (Köffel *et al*, 2005).

In the course of a systematic search for steryl ester hydrolase activities, we uncovered an unidentified [^{14}C]cholesterol-labeled derivative in yeast cells lacking the putative esterase/lipase *YGR263c*, hereafter referred to as *SAY1* (steryl deacetylase). This cholesterol derivative was identified as cholesteryl acetate, indicating that *SAY1* is required for the deacetylation of acetylated sterols. Formation of the cholesteryl acetate requires *ATF2*, an acetyltransferase. *ATF2* and *SAY1* thus form two components that control a novel sterol acetylation/deacetylation cycle. Remarkably, lack of *SAY1* results in the export of acetylated cholesterol into the culture media. This lipid export requires a functional secretory pathway, indicating that the transport of sterol acetate is vesicle mediated. These observations suggest that the substrate specificity of Say1 controls which acetylated sterols are deacetylated and hence retained, and which acetylated sterols are exported and hence potentially detoxified. In agreement with this proposition, we find that the steroid precursor pregnenolone is rapidly acetylated in a reaction that depends on Atf2, but that pregnenolone acetate is not subject to deacetylation by Say1 and hence is exported into the culture media.

Results

Accumulation of cholesteryl acetate in a yeast mutant lacking SAY1

In a screen to identify enzymes that catalyze steryl ester hydrolysis, heme-deficient strains bearing deletions of candidate esterase/lipase genes were labeled with [^{14}C]cholesterol and mobilization of steryl esters was monitored (Köffel *et al*,

*Corresponding author. Department of Medicine, Division of Biochemistry, Institute of Biochemistry, University of Fribourg, Chemin du Musée 5, Fribourg 1700, Switzerland. Tel.: +41 26 300 8654; Fax: +41 26 300 9735; E-mail: roger.schneiter@unifr.ch
[1]Present address: Institute of Immunology, Medical University Vienna, Lazarettgasse 19, Wien 1090, Austria

2005). Heme deficiency is required for cells to take up exogenous sterols under aerobic conditions (Lewis *et al*, 1985). The relative content of free cholesterol and steryl esters was then examined by thin-layer chromatography (TLC). This analysis revealed the presence of a novel [^{14}C]cholesterol-labeled band in cells lacking *YGR263c/SAY1* (Figure 1A). This previously unidentified sterol derivative comprised approximately 25% of the total [^{14}C]cholesterol-labeled lipids present in the *say1*Δ mutant, but was hardly detectable in wild-type cells, thus indicating that *SAY1* is required to prevent its accumulation in wild-type cells. Mild base treatment of this sterol derivative showed that it was susceptible to hydrolysis of an ester bond, indicating that it contains [^{14}C]cholesterol esterified to an unidentified substituent. This substituent confers lower relative mobility and thus is less hydrophobic than long-chain fatty acids in steryl esters (Figure 1B). This novel cholesterol derivative has been identified as cholesterol acetate by comigration analysis with commercial cholesterol acetate (Figure 1C), and confirmed independently by mass spectrometry (Supplementary Figure S1). These results thus show that *say1*Δ-mutant cells accumulate cholesterol acetate.

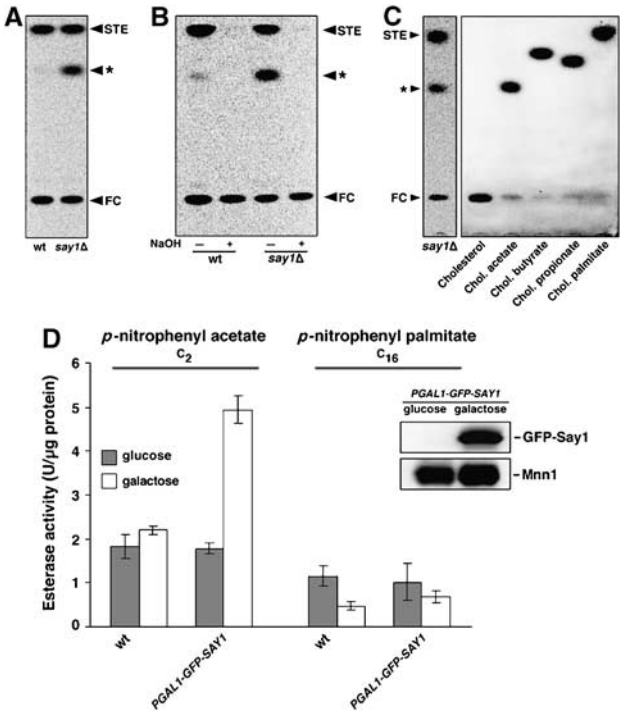

Figure 1 *SAY1* encodes a cholesterol acetate deacetylase. (**A**) An unidentified cholesterol derivative in *say1*Δ-mutant cells. Heme-deficient wild-type (YRS1849) and *say1*Δ-mutant (YRS1853) cells were labeled with [^{14}C]cholesterol for 16 h. Lipids were extracted, separated by TLC, and visualized using a phosphorimager. FC, free cholesterol; STE, steryl ester; the star indicates the unidentified lipid made in cells lacking *SAY1*. (**B**) Mild-base sensitivity of the unidentified lipid. Lipids isolated from [^{14}C]cholesterol-labeled heme-deficient *say1*Δ-mutant cells were deacetylated by incubation with (+) or without (−) 0.1 M NaOH and separated by TLC. (**C**) Comigration of the unidentified lipid with cholesterol acetate. Cholesteryl esters of various chain lengths were separated by TLC, along with radiolabeled lipids from the heme-deficient *say1*Δ mutant. Lipids were visualized by iodine staining and phosphorimaging. (**D**) Deacetylase activity of Say1 *in vitro*. Wild-type (YRS1533) and GFP-Say1-expressing (YRS2211) cells were grown either under repressing (glucose) or inducing (galactose) conditions, and membranes were assayed for deacetylase activity against *p*-nitrophenyl acetate or *p*-nitrophenyl palmitate. Values represent means and standard deviation of three independent determinations. A western blot analysis showing the overexpression of GFP-Say1 in galactose-containing media and its repression in media containing glucose is shown in the inset. Protein loading was monitored using an antibody against the α-1,3-mannosyltransferase, Mnn1.

Since *say1*Δ-mutant cells accumulate cholesterol acetate, we tested if Say1 could function as a deacetylase. Cells overexpressing Say1 from a galactose-inducible promoter exhibited a ~2.5-fold higher *in vitro* activity to hydrolyze *p*-nitrophenyl acetate compared with wild-type cells (Figure 1D). Furthermore, this increased activity was specific for short *p*-nitrophenyl esters, as activity against *p*-nitrophenyl palmitate of *say1*Δ-mutant cells was comparable to that of wild-type cells. These observations are consistent with Say1 being required for deacetylation of cholesterol acetate *in vivo*.

Say1 has homology to mammalian deacetylases

Say1 contains a putative esterase/lipase domain from amino acid 156 to 375 that is predicted to form a canonical α/β-hydrolase fold (Derewenda and Derewenda, 1991). The protein contains a possible N-terminal transmembrane domain and three potential *N*-linked glycosylation sites. PSI-BLAST analysis revealed high homology of Say1 with two predicted ORFs from *Caenorhabditis elegans* (T09B9.1 and F27C8.6) and with human arylacetamide deacetylase (AADAC) and its homologues AADACL1, AADACL2, and AADACL4 (Probst *et al*, 1994). Multiple alignment of these sequences with Say1 revealed that the lipase consensus motif GXSXG is conserved in Say1 and its metazoan homologue as GDSAG, with a central nucleophilic Ser at position 250 and conservation of the HGGG oxyanion hole motif at position 176 of Say1 (Figure 2A). These two sequence motifs are signatures for the prokaryotic lipolytic enzymes classified as belonging to family IV, which also shows high similarity to mammalian hormone-sensitive lipases (Hemila *et al*, 1994; Arpigny and Jaeger, 1999). Mutations of the putative active site Ser at position 250 of Say1 to Ala resulted in the accumulation of acetylated sterols, indicating that this Ser is required for Say1 function (Figure 2B). Loss of Say1p activity due to the exchange of Ser 250 for Ala does not affect protein abundance as indicated by western blot analysis of an myc-epitope tagged version of Say1p, consistent with the proposition that Ser 250 forms the active site residues of Say1p (Figure 2C). Assignment of Ser 250 as the active-site nucleophile is consistent with the assignment of active-site residues in the mouse AADACL1 that is based on homology modeling (Nomura *et al*, 2006).

Say1 is an integral membrane protein with the putative active site in the ER lumen

To determine whether Say1 is membrane-anchored and to examine its membrane topology and subcellular localization, N-terminally GFP-tagged Say1 under the control of the *GAL1/10* promoter and C-terminally myc-tagged versions of Say1 were generated. Both fusion proteins were functional, as cells expressing these proteins displayed no detectable accumulation of cholesteryl acetate when labeled with [^{14}C]cholesterol (Figure 3A). Differential fractionation revealed that GFP-Say1 and Say1-myc were enriched in the 13 k membrane fraction, indicating that Say1 is membrane associated (Figure 3B). Say1 was released from membranes only upon treatment

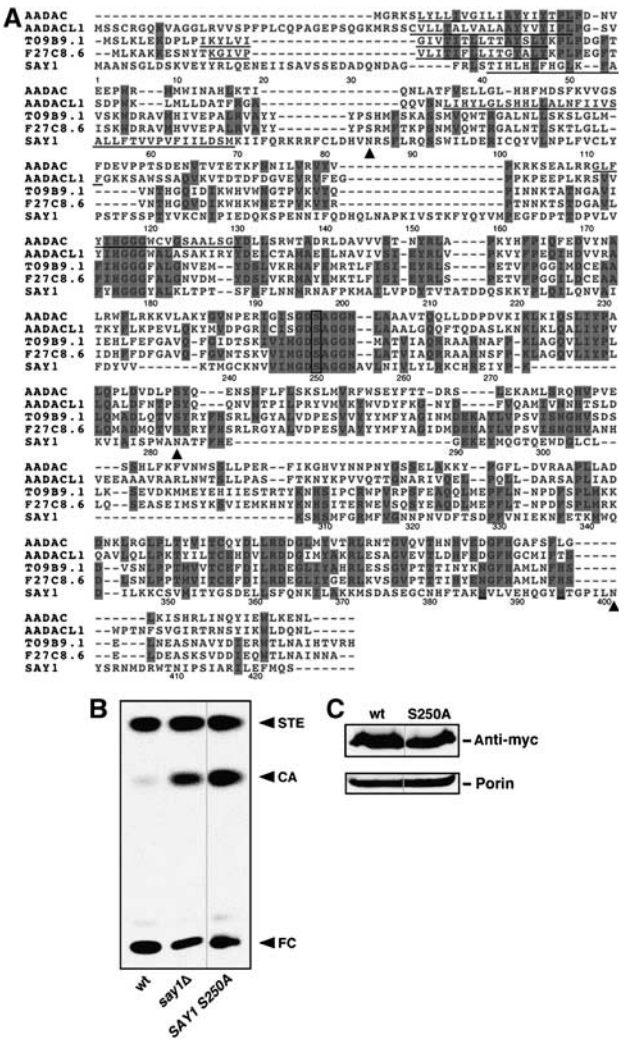

Figure 2 Homology of Say1 to human arylacetamide deacetylase. (**A**) Sequence alignment of Say1 with AADAC, and its homologue AADACL1, and two predicted ORFs from *C. elegans*, T09B9.1 and F27C8.6. Predicted N-terminal transmembrane domains are indicated by horizontal lines. Possible *N*-linked glycosylation sites on Say1 are indicated by filled arrowheads (85, 283, 401). The putative active-site residue Ser 250 within the conserved GDSAG motif is boxed. Sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/) and regions of homology were drawn by Boxshade (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html). (**B**) Ser at position 250 is required for Say1 function. Heme-deficient wild-type (YRS1849), *say1*Δ (YRS1853), and a *say1* point-mutant with Ser 250 exchanged for Ala (S250A, YRS2857) were labeled with [^{14}C]cholesterol for 16 h. Lipids were extracted and separated by TLC. FC, free cholesterol; STE, steryl ester; CA, cholesterol acetate. (**C**) Expression levels of wild type and mutant Say1p. Cells expressing myc-tagged wild-type (YRS2528) or S250A-mutant version of Say1p (YRS2776) were cultivated and expression levels of Say1p were assessed by western blotting. Protein loading was controlled by blotting with an antibody against the mitochondrial porin.

with 1% SDS or 1% Triton X-100, indicating that Say1 is an integral membrane protein (Figure 3C). Accessibility of the N- and C-terminal tags to degradation by proteinase K revealed that the N-terminal GFP was readily hydrolyzed. At the same time, the ER lumenal Kar2 was protease protected, indicating intactness of the membrane seal. The myc-tagged C-terminus of Say1, on the other hand, resisted protease treatment, but became protease sensitive in the presence of Triton X-100, consistent with a lumenal localization of the C-terminus of Say1 (Figure 3D). Say1 contains three asparagines that could potentially be subjected to *N*-linked glycosylation. To determine whether Say1 is *N*-glycosylated, we treated membranes with endoglycosidase H. This analysis revealed that none of these consensus residues for *N*-linked glycosylation is actually glycosylated in Say1, which does neither confirm nor invalidate the topology model of Say1, as glycosylation of Asn–Xaa–Ser/Thr sequons is not obligatory (Figure 3E; Nilsson and von Heijne, 2000).

Analysis of the subcellular localization of Say1 by fluorescence microscopy showed colocalization of GFP-Say1 with the ER marker Kar2-mRFP-HDEL (Gao *et al*, 2005). Immunofluorescence microscopy revealed colocalization of Say1-myc with Sec61, a subunit of the ER translocon, indicating that Say1 is an ER-localized protein (Figure 3F). ER localization of Say1 is consistent with its fractionation properties on an Accudenz density gradient on which the protein cofractionates with the ER lumenal chaperone Kar2 but not with the plasma membrane localized H^+-ATPase, Pma1 (Figure 3G). Taken together, these observations indicate that Say1 is a type II integral membrane protein with its putative active site Ser exposed to the ER lumen (Figure 3H). Deacetylation of cholesterol acetate thus occurs in the ER lumen.

ATF2 is required for the formation of acetylated sterols

As acetylated cholesterol is detectable only in cells lacking *SAY1*, we looked at the acetyltransferase(s) required for its formation. Yeast contains two genes encoding alcohol *O*-acetyltransferases, *ATF1* and *ATF2* (Mason and Dufour, 2000). Atf1 and Atf2 control the production of a broad range of volatile esters that are important for the fruity flavor of fermented beverages (Verstrepen *et al*, 2003). Atf2 has previously been shown to acetylate the steroid precursor, pregnenolone, and it has been proposed that this acetylation is important for detoxification and excretion of steroids (Cauet *et al*, 1999). To determine whether *ATF1* and/or *ATF2* are required for the formation of cholesteryl acetate *in vivo*, heme-deficient *atf1*Δ *say1*Δ and *atf2*Δ *say1*Δ double mutant cells were generated. These were labeled with [^{14}C]cholesterol and lipids were analyzed for the presence of cholesteryl acetate. While cholesteryl acetate was present in cells lacking *ATF1*, no acetylated cholesterol was detectable in cells lacking *ATF2*, indicating that *ATF2* is required for the formation of acetylated sterols (Figure 4A).

Atf2 contains two weakly predicted transmembrane domains located between amino acids 313–339 and 478–493. To determine the cellular site of sterol acetylation, the localization of Atf2 was examined in more detail, using N-terminal GFP fused to Atf2 under control of a *GAL1/10* promoter. Expression of the GFP-tagged Atf2 resulted in a functional protein as indicated by the presence of acetylated cholesterol upon induction of GFP-Atf2 expression (Figure 4B). Furthermore, overexpression of GFP-Atf2 resulted in the appearance of acetylated sterols even when Say1 was functional, indicating that the activities of Atf2 and Say1 in wild-type cells must be balanced to prevent the accumulation of acetylated sterols (Figure 4B).

Differential fractionation indicated that GFP-Atf2 is membrane associated as it was enriched in both the 13 and 30 k membrane pellets (Figure 4C). The protein was solubilized by

detergent treatment only, indicating that Atf2 is an integral membrane protein (Figure 4D). Proteinase K treatment of N- and C-terminally tagged versions of Atf2 indicates that both termini of the protein are protease protected in the absence of detergent, but protease sensitive in the presence of detergent, consistent with a lumenal orientation of these termini. The appearance of N- and C-terminal cleavage fragment is furthermore consistent with a protease attack within an exposed cytosolic loop (Figure 4E). Endoglycosidase H treatment revealed no altered mobility of GFP-Atf2, indicating that the protein is not glycosylated (Figure 4F). Fluorescence microscopy is consistent with an ER localization of GFP-Atf2 (Figure 4G). Taken together, this analysis indicates that Atf2 is required for acetylation of sterols and that Atf2 is an integral membrane protein of the ER with at least two transmembrane domains with both termini oriented toward the lumenal compartment (Figure 4H). This topology places the heptapeptide WRLICLP that is conserved between Atf1 and Atf2 and that has been proposed to be part of the active site of the two acetyltransferases into the ER lumen (Mason and Dufour, 2000), suggesting that sterol acetylation occurs in the lumen of the ER.

Acetylated sterols are exported

Given that Atf2 and Say1 catalyze the acetylation and deacetylation respectively of exogenously supplied cholesterol, we next asked what the physiological function of this acetylation might be. Since both acetylation and deacetylation of sterols occurs in the ER and *say1*Δ-mutant cells accumulate sterol acetate but do not display any ER-related phenotype (data not shown), we examined the possibility that acetylation of sterols could be a signal for export of the lipid. Therefore, we examined whether *say1*Δ cells secrete acetylated cholesterol into the culture media. Following labeling of *say1*Δ with [^{14}C]cholesterol, lipids from the cell pellet and the growth media was examined. This analysis revealed that the acetylated cholesterol was present in the culture supernatant of *say1*Δ-mutant cells, indicating that the acetylation of sterols may determine their excretion (Figure 5A). The fact that the medium is devoid of long-chain steryl esters indicates that the presence of acetylated cholesterol is not simply due to cell lysis. Excretion of cholesterol acetate is selective as indicated by the fact that more than 50% of the total cholesterol acetate, but only 3% of steryl esters is found in the culture supernatant (Figure 5B). Export of acetylated cholesterol is stimulated by the presence of ergosterol in the media (Supplementary Figure S2) and requires ATP, but is independent of ongoing protein synthesis (data not shown), indicating that it is an active process rather than occurring by

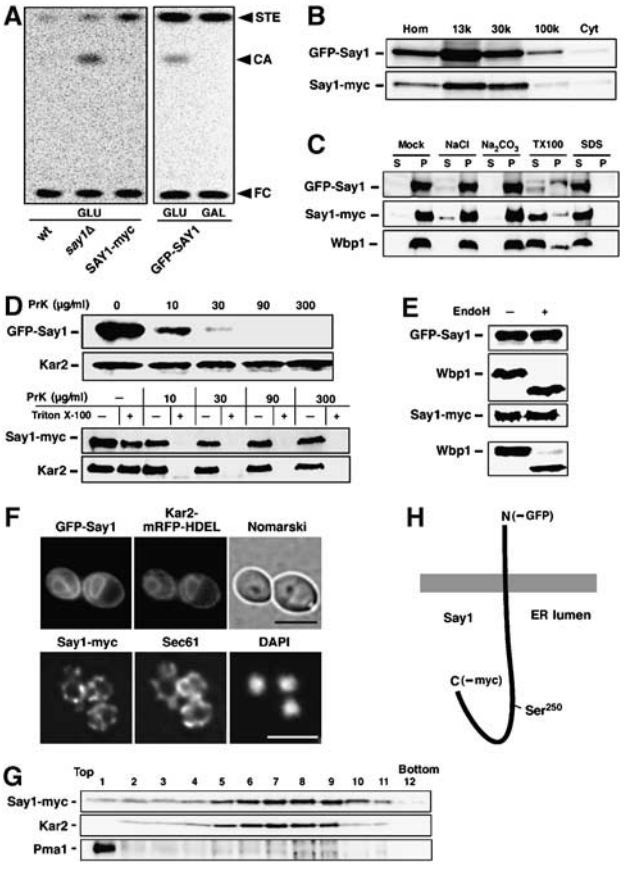

Figure 3 Say1 is an integral membrane protein with the putative active site toward the ER lumen. (**A**) Functionality of epitope-tagged Say1. Heme-deficient cells expressing chromosomally tagged versions of Say1-myc (YRS2529) or GFP-Say1 (YRS2212) were cultivated in media containing either glucose or galactose and labeled with [^{14}C]cholesterol. Lipids were extracted and analyzed by TLC together with lipids from heme-deficient wild-type and *say1*Δ-mutant cells. FC, free cholesterol; CA, cholesteryl acetate; STE, steryl ester. (**B**) Say1 is membrane associated. Homogenates (Hom) from cells expressing GFP-tagged Say1 (YRS2211) or Say1-myc (YRS2528) were fractionated by differential centrifugation to yield 13 000 *g* (13 k), 30 000 *g* (30 k), and 100 000 *g* (100 k) membrane pellets and cytosolic supernatants (Cyt). A 10 μg weight of proteins from each fraction was separated by electrophoresis, blotted, and probed with anti-GFP and anti-myc antibodies, respectively. (**C**) Say1 is an integral membrane protein. Membranes from cells expressing GFP- or myc-tagged Say1 were incubated with 1 M NaCl, 0.1 M Na_2CO_3, 1% Triton X-100 (TX100), 1% SDS, or buffer alone (Mock) for 30 min at 4°C and then centrifuged at 13 k for 15 min to yield soluble (S) and pellet (P) fractions. Proteins were precipitated by TCA, separated by electrophoresis, and probed for the presence of the epitopes. Fractionation of the integral membrane protein Wbp1 is shown as control. (**D**) Proteinase K sensitivity of N- and C-terminal tags of Say1. Membranes from cells expressing GFP-Say1 were incubated with the indicated concentrations of proteinase K (PrK) in the presence (+) or absence (−) of 0.1% Triton X-100 for 30 min on ice, proteins were precipitated, separated by electrophoresis, and probed for the presence of the epitopes and for Kar2. (**E**) Say1 is not glycosylated. Extracts from cells expressing GFP- or myc-tagged Say1 were incubated with (+) or without (−) endoglycosidase H (EndoH) at 37°C for 16 h. Proteins were precipitated, separated by electrophoresis, and probed for the presence of the epitopes and for the β-subunit of the oligosaccharyltransferase, Wbp1. (**F**) Localization of GFP-Say1 and Say1-myc by fluorescence microscopy. Cells expressing GFP-Say1 and the ER marker Kar2-mRFP-HDEL (YRS3052) were cultivated in galactose-containing media for 8 h, and GFP and RFP fluorescence was analyzed by fluorescence microscopy. Cells expressing Say1-myc (YRS2528) were fixed and processed for immunofluorescence microscopy using a mouse anti-myc and a rabbit antibody against the translocon subunit, Sec61. Nuclei were stained with DAPI and cells were visualized using Nomarski optics. Bar, 5 μm. (**G**) Say1 cofractionates with the ER marker protein Kar2. Membranes from cells expressing Say1-myc were fractionated on an Accudenz density gradient and the presence of Say1-myc was detected by western blotting with an anti-myc antibody. The distribution of marker proteins in individual fractions was determined by probing with antibodies against the ER lumenal chaperone Kar2, and the plasma membrane proton pumping ATPase, Pma1. (**H**) Schematic representation of the membrane topology of Say1.

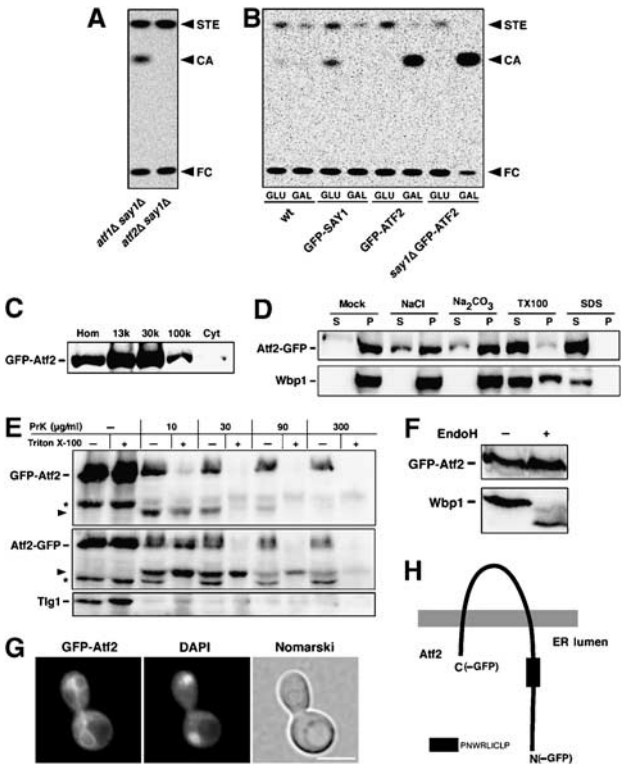

Figure 4 Atf2, an ER-localized acetyltransferase controls sterol acetylation. (**A**) *ATF2* is required for the formation of acetylated sterols. Heme-deficient *atf1*Δ *say1*Δ- (YRS2209) and *atf2*Δ *say1*Δ-mutant (YRS2136) cells were labeled with [^{14}C]cholesterol, and lipids were extracted and analyzed by TLC. FC, free cholesterol; CA, cholesteryl acetate; STE, steryl ester. (**B**) Overexpression of a GFP-tagged version of Atf2 results in the accumulation of acetylated cholesterol. Heme-deficient wild-type (YRS1849), *GFP-SAY1*- (YRS2212), *GFP-ATF2*- (YRS2538), and *say1*Δ *GFP-ATF2*-mutant (YRS2539) cells were cultivated in media containing glucose or galactose and labeled with [^{14}C]cholesterol for 16 h. Lipids were extracted and analyzed by TLC. FC, free cholesterol; CA, cholesteryl acetate; STE, steryl ester. (**C**) Atf2 is membrane associated. Homogenates (Hom) from cells expressing GFP-Atf2 (YRS2537) were fractionated by differential centrifugation to yield 13 000 *g* (13 k), 30 000 *g* (30 k) and 100 000 *g* (100 k) membrane pellets and cytosolic supernatants (Cyt). A 10 μg weight of proteins from each fraction was separated by electrophoresis, blotted, and probed with an anti-GFP antibody. (**D**) Atf2 is an integral membrane protein. Membranes from cells expressing Atf2-GFP (YRS2483) were incubated with 1 M NaCl, 0.1 M Na_2CO_3, 1% Triton X-100 (TX100), 1% SDS, or buffer alone (Mock) for 30 min at 4°C and then centrifuged at 13 k for 15 min to yield soluble (S) and pellet (P) fractions. Proteins were precipitated by TCA, separated by electrophoresis, and probed with an antibody against GFP or the β-subunit of the oligosaccharyltransferase, Wbp1. (**E**) Proteinase K sensitivity of the N- and C-terminal tags of Atf2. Membranes from cells expressing GFP-Atf2 or Atf2-GFP were incubated with the indicated concentrations of proteinase K (PrK) in the presence (+) or absence (–) of 0.1% Triton X-100 for 30 min on ice; proteins were precipitated, separated by electrophoresis, and probed for the presence of the GFP-epitope and for the t-SNARE, Tlg1. The star indicates cross-reacting material of unknown identity. The arrowhead indicates N- and C-terminal cleavage products. (**F**) Atf2 is not glycosylated. Extracts from cells expressing GFP-Atf2 were treated (+) or not (–) with endogylcosidase H (EndoH). Proteins were precipitated, separated by electrophoresis, and probed with antibodies against GFP and Wbp1. (**G**) Localization of GFP-Atf2 by fluorescence microscopy. Cells expressing GFP-Atf2 were cultivated in galactose-containing media for 8 h and GFP fluorescence was analyzed by fluorescence microscopy. Nuclei were stained with DAPI and cells were visualized using Nomarski optics. Bar, 5 μm. (**H**) Schematic representation of the membrane topology of Atf2, with a conserved sequence indicated by a box.

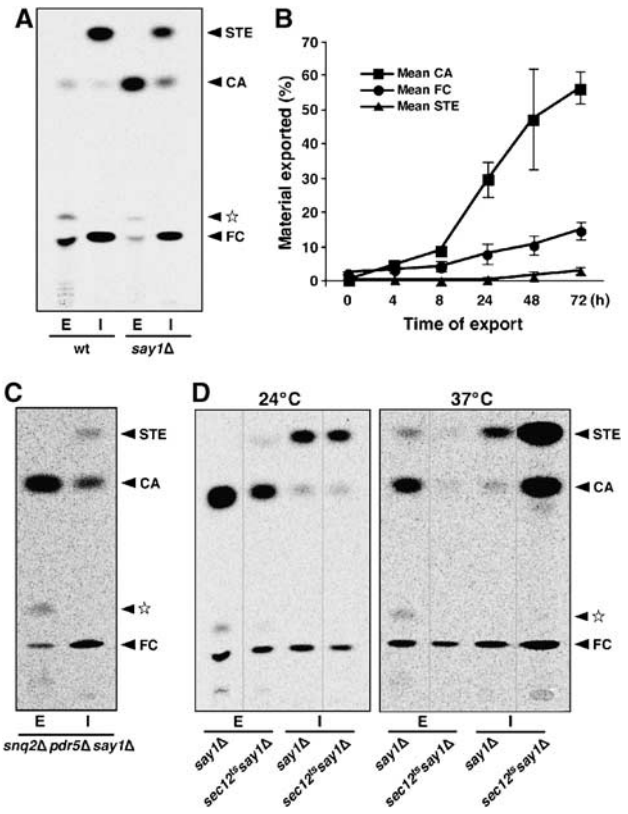

Figure 5 Acetylated sterols are secreted. (**A**) Heme-deficient wild-type (YRS1849) and *say1*Δ-mutant (YRS1853) cells were labeled with [^{14}C]cholesterol for 16 h, diluted into fresh media containing cold ergosterol, and cultivated for 6 h. Lipids were extracted from the cell pellet (I) and the culture media (E) and analyzed by TLC. FC, free cholesterol; CA, cholesterol acetate; STE, steryl esters; the open star indicates an unidentified secreted sterol derivative. (**B**) Export of cholesterol acetate is selective. Heme-deficient *say1*Δ-mutant (YRS1853) cells were labeled with [^{14}C]cholesterol for 16 h, diluted into fresh media containing cold ergosterol, and cultivated for the times indicated. Lipids were extracted from the cell pellet and the culture media, analyzed by TLC, and quantified. The proportion of CA, FC, and STE that is exported into the culture media is plotted as a function of time. (**C**) Sterol excretion is independent of Pdr5 and Snq2. Heme-deficient *pdr5*Δ *snq2*Δ *say1*Δ-mutant (YRS2495) cells were labeled with [^{14}C]cholesterol diluted into fresh media and cultivated at 24°C for 6 h. Lipids were extracted from the cell pellet (I) and the culture media (E) and analyzed by TLC. FC, free cholesterol; CA, cholesterol acetate; STE, steryl esters; the open star indicates an unidentified exported sterol derivative. (**D**) Sterol export requires vesicular transport out of the ER. Heme-deficient *say1*Δ- (YRS1853) and *sec12^{ts}say1*Δ-mutant (YRS2655) cells were labeled with [^{14}C]cholesterol diluted into fresh media and cultivated at either 24 or 37°C for 6 h. Lipids were extracted from the cell pellet (I) and the culture media (E) and analyzed by TLC. FC, free cholesterol; CA, cholesterol acetate; STE, steryl esters; the open star indicates an unidentified exported sterol derivative.

passive diffusion of acetylated cholesterol through the cell membrane.

Export of the acetylated form of the steroid precursor pregnenolone has previously been suggested to depend on two plasma membrane-localized ABC transporters, Pdr5p and Snq2p, since pregnenolone toxicity is increased in *pdr5*Δ and *snq2*Δ-mutant cells (Kralli *et al*, 1995; Kolaczkowski *et al*, 1996; Mahe *et al*, 1996; Cauet *et al*, 1999). Direct examination of a possible role of the two ABC transporters, in export of sterol acetate revealed that a *pdr5*Δ

*snq2*Δ *say1*Δ triple mutant, exported cholesterol acetate into the culture supernatant similar to a *say1*Δ single mutant, indicating that these two ABC transporters are not required for the export of cholesterol acetate (Figure 5C).

Since cholesterol acetate is formed in the ER lumen, we next tested whether export of this lipid requires ongoing vesicular transport from the ER. Therefore, vesicle formation was conditionally blocked using a temperature-sensitive allele of *SEC12*, *sec12*^{ts}. *SEC12* encodes the guanine nucleotide exchange factor for Sar1p and is required for the assembly of the COPII coat on the ER membrane (Barlowe and Schekman, 1993). *sec12*^{ts} *say1*Δ-mutant cells secreted cholesterol acetate into the culture supernatant at the permissive temperature. When shifted to the non-permissive temperature of 37°C, however, cholesterol acetate was absent from the supernatant, indicating that export of cholesterol acetate depends on ongoing membrane transport out of the ER (Figure 5D). Lack of export of cholesterol acetate is not simply due to lack of its synthesis, as cholesterol acetate accumulates intracellularly under conditions that block secretion. Taken together, these results indicate that cholesterol acetylation/deacetylation cycle controls the export of acetylated sterols through the secretory pathway.

Formation of cholesterol acetate was not impaired in cells lacking the two acyl-CoA:sterol acyltransferases, Are1 and Are2 (Yang *et al*, 1996; Yu *et al*, 1996), indicating that formation of long-chain steryl esters is not a prerequisite for acetylation (Supplementary Figure S3A). *are1*Δ *are2*Δ *say1*Δ triple mutant cells, however, exported cholesterol acetate less efficiently than *say1*Δ-mutant cells (Supplementary Figure S3B). The fact that deletion of either Say1 or Atf2 in an *are1*Δ *are2*Δ double mutant background results in viable triple mutants indicates that acetylation and export of sterols does not become essential in cells that lack the capacity to buffer sterols by forming long-chain steryl esters (Supplementary Figure S4).

Export of pregnenolone requires Atf2 but is independent of Say1

Given that acetylated cholesterol but not non-acetylated cholesterol is secreted from the cells, substrate acetylation and/or deacetylation is likely to control sterol export. To examine whether substrate recognition could play a role in discriminating between sterols that need to be secreted from those that are retained, we tested whether the steroid precursor pregnenolone or aberrant endogenously synthesized sterols are substrates of the acetylation/deacetylation cycle. Therefore, we first examined pregnenolone acetylation and export in wild-type, *say1*Δ- and *atf2*Δ-mutant cells. Cells were incubated with radiolabeled pregnenolone for 2 h, lipids were extracted from the cell pellet and culture supernatant, and examined by TLC. This analysis revealed that pregnenolone was rapidly acetylated in a reaction that depends on Atf2 and exported from the cells (Figure 6A). These observations are consistent with the report that Aft2 is an acetyl-CoA:pregnenolone acetyltransferase and that pregnenolone acetate is exported from cells (Cauet *et al*, 1999). The fact that wild-type and *say1*Δ-mutant cells had comparable levels of pregnenolone acetate indicates that pregnenolone acetate is not subject to deacetylation by Say1. This could be either because pregnenolone acetate has no access to Say1 or because it is not a substrate for Say1. In the absence of

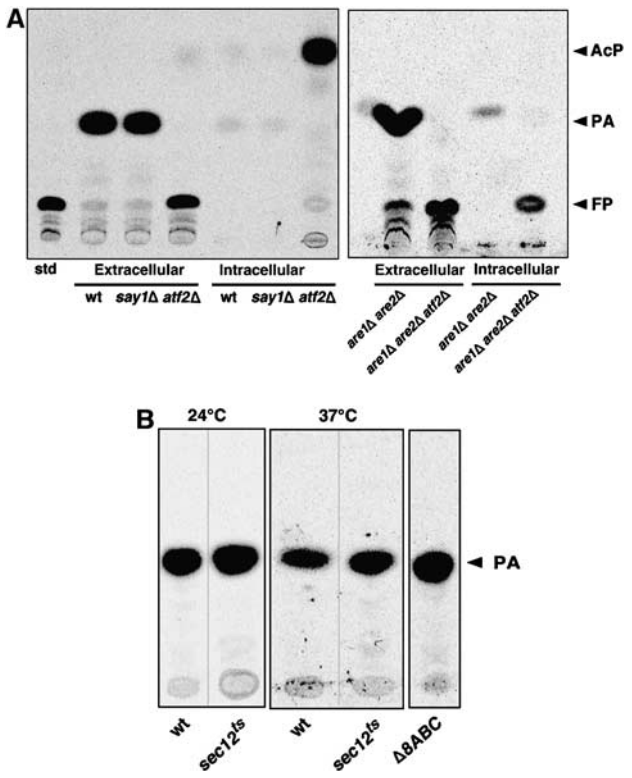

Figure 6 Sterol acetylation/deacetylation controls the export of steroids. (**A**) Atf2 is required for acetylation and excretion of pregnenolone. Wild-type (YRS1533), *say1*Δ- (YRS2550), *atf2*Δ- (YRS2551), *are1*Δ *are2*Δ- (YRS2486), and *are1*Δ *are2*Δ *atf2*Δ-mutant (YRS3279) cells were labeled with [^{3}H]pregnenolone, lipids were extracted from the cell pellet (intracellular), and the culture media (extracellular) and analyzed by TLC. Pregnenolone was loaded as standard (std). FP, free pregnenolone; PA, pregnenolone acetate; AcP, acylated pregnenolone. (**B**) Export of pregnenolone acetate is independent of the secretory pathway. Wild-type (YRS1533), *sec12*^{ts} (YRS1845) and 8ΔABC (YRS2656, lacking *YOR1*, *SNQ2*, *PDR5*, *PDR10*, *PDR11*, *YCF1*, *PDR3*, *PDR15*) transporter-mutant cells were labeled with [^{3}H]pregnenolone for 2 h at either 24 or 37°C, cells were pelleted, and lipids in the culture media were analyzed by TLC. PA, pregnenolone acetate.

Atf2, pregnenolone is acylated with long-chain acyl-CoA in a reaction that depends on the presence of Are1 and Are2, and accumulates intracellularly (Figure 6A). Also, export of pregnenolone acetate is mechanistically distinct from that of cholesterol acetate, as it does not depend on ongoing vesicular transport, as shown by its excretion from *sec12*^{ts} mutants under both permissive and non-permissive conditions, nor is it blocked by the deletion of eight ABC transporters, including Pdr5 and Snq2 (Nakamura *et al*, 2001; Figure 6B). These results are thus consistent with a proofreading function of Say1 in which substrate access or recognition by Say1 determines whether the acetylated sterol is deacetylated and hence retained, or whether it is not deacetylated and thereby exported from the cell.

Further analysis of the *in vivo* substrate specificity of the sterol acetylation and export pathway indicated that sitosterol, progesterone, 7-ketocholesterol, and lanosterol are not subject to acetylation, but that 25-hydroxycholesterol, which inhibits cholesterol biosynthesis in mammalian cells and does not support growth of yeast (Rodriguez *et al*, 1983; Adams *et al*, 2004), behaves like pregnenolone, that is, is

subject to Atf2-dependent acetylation (Supplementary Figure S5).

Endogenously synthesized sterols are subject to the acetylation/deacetylation cycle

To examine whether the acetylation/deacetylation cycle is also operating on endogenously made sterols, we blocked intermediate steps in the biosynthetic pathway for ergosterol and analyzed the formation of acetylated sterol intermediates and their export. Among the viable mutants in the ergosterol biosynthetic pathway tested, we observed that *erg4*Δ mutants displayed accumulation of an aberrant sterol in a *say1*Δ-mutant background, as revealed by labeling endogenously synthesized sterols with tritiated methionine, which labels the methyl group at position C-24 in the side chain of ergosterol. *ERG4* encodes the sterol C-24 reductase that catalyzes the final step in ergosterol biosynthesis (Daum *et al*, 1998). *erg4*Δ mutants thus accumulate ergosta-5,7,22,24(28)-tetraenol, which is modified in a reaction that depends on *ATF2*, and the aberrant sterol formed migrates similar to cholesterol acetate when analyzed by TLC (Figure 7A). The acetylated sterol intermediate produced in *erg4*Δ-mutant cells is exported in the absence of *SAY1*, indicating that Say1 deacetylates it and thus prevents its secretion (Figure 7B). Export of aberrant sterols that are produced under aerobic conditions, however, is much less efficient compared with the export of cholesterol under anaerobic conditions. Taken together, these observations indicate that the sterol acetylation/deacetylation cycle also operates on the aberrant sterols that accumulate endogenously in an *erg4*Δ mutant, and results in the secretion of the acetylated sterol intermediate.

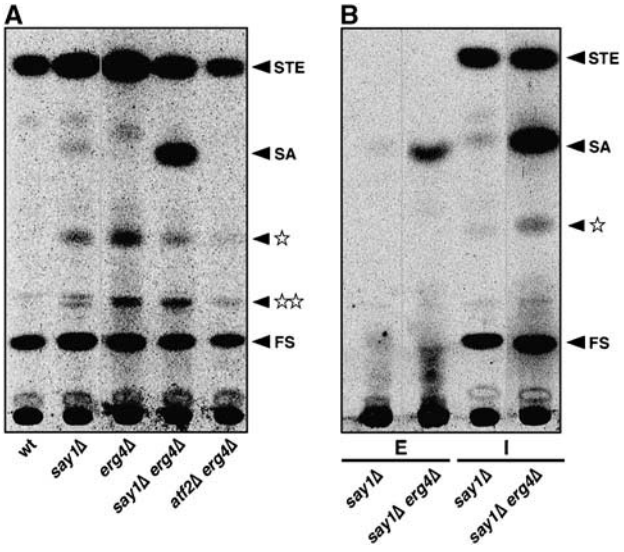

Figure 7 Sterol acetylation/deacetylation controls the export of intermediates in sterol biosynthesis. (**A**) Endogenously synthesized sterols are subject to the acetylation/deacetylation cycle. Wild-type (YRS1533), *say1*Δ- (YRS2550), *erg4*Δ- (YRS2862), *say1*Δ *erg4*Δ- (YRS2846), and *atf2*Δ *erg4*Δ-mutant (YRS2851) cells were labeled with L-[methyl-^{3}H]methionine for 16 h; lipids from the cells were isolated and analyzed by TLC. FS, free sterol; SA, sterol acetate; STE, steryl ester; the open star and double-star indicate lipids of unknown identity. (**B**) Endogenously synthesized sterols are exported. *say1*Δ- (YRS2550) and *say1*Δ *erg4*Δ-mutant (YRS2846) mutant cells were labeled with L-[methyl-^{3}H]methionine for 16 h, lipids from the culture media (extracellular) and from the cells (intracellular) were isolated and analyzed by TLC. FS, free sterol; SA, sterol acetate; STE, steryl ester; the open star indicates a lipid of unknown identity.

Sterol deacetylation is conserved

We next wondered whether this sterol acetylation/deacetylation cycle that controls sterol export in yeast is conserved in metazoans. Say1 shows homology to the arylacetamide deacetylase AADAC and its homologues AADACL1, AADACL2, and AADACL4 in humans. We first tested whether expression of the human homologues in yeast would complement for Say1. Therefore, expression of AADAC and AADACL1 was placed under control of a *GAL1/10* promoter and the proteins were expressed in a *say1*Δ *hem1*Δ-mutant background. Cells were then labeled with [^{14}C]cholesterol and lipids were analyzed by TLC. This analysis revealed that expression of AADAC but not of AADACL1 prevented the accumulation of cholesterol acetate, indicating that AADAC provides deacetylase activity against cholesterol acetate when expressed in yeast (Figure 8).

Say1 and Atf2 are required for growth in the presence of eugenol and affect pregnenolone toxicity

To examine whether the sterol acetylation cycle might have a function in detoxification of cells from potentially toxic steroid-like compounds, we examined the growth of *say1*Δ and *atf2*Δ-mutant cells in the presence of various naturally occurring hydrophobic and steroid-like compounds. This analysis revealed a strong growth advantage of cells expressing Say1 and Atf2 on media containing eugenol (Figure 9A). Eugenol is a member of the allylbenzene class of compounds that is present in clove oil, nutmeg, cinnamon, and bay leaf, and is used as local antiseptic and anesthetic. The fact that both *say1*Δ and *atf2*Δ-mutant cells are sensitive against

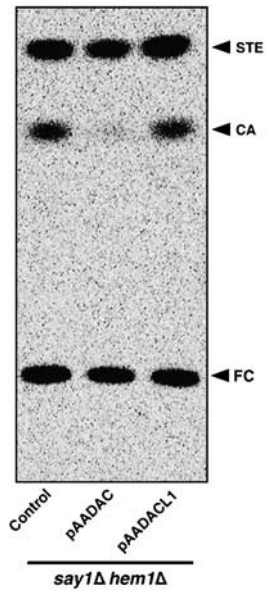

Figure 8 Sterol deacetylation is conserved. Expression of the human arylacetamide deacetylase AADAC rescues *say1*Δ. Heme-deficient *say1*Δ bearing an empty plasmid (YRS2890), an expression plasmid containing AADAC (YRS2985), or AADACL1 (YRS2891) were cultivated in galactose-containing media and labeled with [^{14}C]cholesterol. Lipids were extracted from the cells and analyzed by TLC. FC, free cholesterol; CA, cholesteryl acetate; STE, steryl ester.

eugenol indicates that the full acetylation/deacetylation cycle must be operating for growth in the presence of eugenol, which is consistent with a possible function of this cycle in lipid detoxification.

Export of the acetylated form of the steroid precursor pregnenolone has previously been suggested to depend on two plasma membrane-localized ABC transporters, Pdr5p and Snq2p, since pregnenolone toxicity is increased in *pdr5*Δ- and *snq2*Δ-mutant cells (Kralli *et al*, 1995; Kolaczkowski *et al*, 1996; Mahe *et al*, 1996; Cauet *et al*, 1999). Pregnenolone toxicity in these ABC transporter mutants, however, depends on a tryptophan auxotrophy of the genetic background, as *pdr5*Δ *snq2*Δ *TRP1* double mutant cells do not show any steroid-dependent growth phenotype (Vico *et al*, 2002). To examine whether Say1 and Atf2 affect the sensitivity of cells to grow in the presence of pregnenolone, we generated an isogenic set of strains that are either tryptophan prototrophic (*TRP1*) or auxotrophic (*trp1*Δ) and tested their growth in the presence of pregnenolone. Consistent with the notion that pregnenolone toxicity depends on tryptophan auxotrophy, we find that loss of *ATF2* rendered cells sensitive to pregnenolone in a tryptophan auxotrophic but not in a prototrophic background (Figure 9B). Cells lacking long-chain steryl esters (*are1*Δ *are2*Δ double mutants), on the other hand, are not more pregnenolone sensitive than a wild type, indicating that esterification with long-chain fatty acids and intracellular storage of the resulting steryl esters does not act to buffer the toxic effect of pregnenolone. The tryptophan dependence of pregnenolone toxicity is likely linked to the fact that surface transport of the tryptophan permease, Tat2, is dependent on membrane sterols, which renders tryptophan auxotrophic strains more sensitive to sterol alterations (Umebayashi and Nakano, 2003). Overexpression of Atf2 from a *GAL1* promoter in a tryptophan auxotrophic background, on the other hand, rendered cells more resistant against pregnenolone, whereas overexpression of Say1 rendered them sensitive against pregnenolone (Figure 9C). Taken together, these data show that a functional acetylation cycle affects growth in the presence of toxic lipids, and indicates that the lipid acetylation cycle might function as a detoxification pathway.

Discussion

In the course of a systematic analysis of the role of candidate lipases in steryl ester hydrolysis, we here uncovered a novel cholesterol-derived lipid, cholesterol acetate, in cells lacking *SAY1*, a hydrolase that converts cholesterol acetate into free cholesterol. Even though *SAY1* is not essential, it is conserved in fungi and has homologues in metazoans, suggesting that its metabolic function has been conserved. Say1 is a type II integral membrane protein of the ER with the presumed catalytic site in the ER lumen. The acetyltransferase required for synthesis of acetylated sterols is encoded by *ATF2*, a sterol *O*-acetyltransferase that is bound to the ER membrane and acetylates the steroid precursor pregnenolone (Cauet *et al*, 1999; Mason and Dufour, 2000). *SAY1* and *ATF2* are thus two components of a novel sterol acetylation/deacetylation cycle. This cycle operates on endogenously synthesized ergosterol precursors as well as on exogenously supplied steroids, and could serve to detoxify the cells of steroid-like compounds and hydrophobic phytochemicals such as flavonoids that are present in plants and fruits, one of the natural environments of yeast, as indicated by the sensitivity of *say1*Δ- and *atf2*Δ-mutant cells against eugenol (Figures 9 and 10). Examination of the expression of Say1 and Atf2 under aerobic and anaerobic conditions, however, revealed no oxygen-dependent regulation of the steady-state levels of the two enzymes,

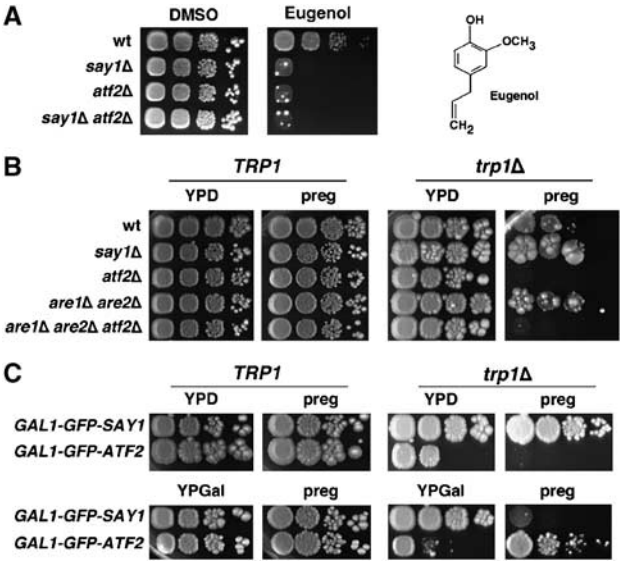

Figure 9 Say1 and Atf2 function in lipid detoxification. (**A**) Say1 and Atf2 are required for growth in the presence of eugenol. Wild-type (YRS1533), *say1*Δ- (YRS2550), *atf2*Δ- (YRS2551), and *say1*Δ *atf2*Δ-mutant (YRS2133) cells were serially diluted 10-fold and stamped on YPD plates containing DMSO or eugenol (1.2 mM) and the plates were incubated at 30°C for 4 days. (**B**) Lack of *ATF2* in a tryptophan auxotrophic background renders cells hypersensitive towards pregnenolone. Tryptophan prototrophic or auxotrophic wild-type (YRS1533, YRS3019), *say1*Δ- (YRS2550, YRS3275), *atf2*Δ- (YRS2551, YRS3276), *are1*Δ *are2*Δ- (YRS2486, YRS3018), and *are1*Δ *are2*Δ *atf2*Δ-mutant (YRS3279, YRS3281) cells were serially diluted 10-fold and spotted on YPD plates and YPD plates containing pregnenolone (preg, 10 μg/ml) and plates were incubated at 30°C for 4 days. (**C**) Overexpression of *ATF2* in a tryptophan auxotrophic background renders pregnenolone resistant, whereas overexpression of Say1 renders cells pregnenolone sensitive. Tryptophan prototrophic or auxotrophic cells expressing GFP-Say1 (YRS2211, YRS3277) or GFP-Atf2 (YRS2537, YRS3278) from a *GAL1* promoter were serially diluted 10-fold and spotted on YPD and YPGal plates in the presence or absence of pregnenolone (10 μg/ml) and plates were incubated at 30°C for 4 days.

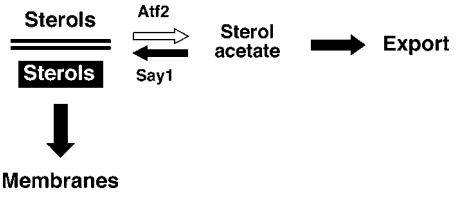

Figure 10 Model for the sterol proofreading and detoxification activity of the acetylation/deacetylation cycle. Sterols and steroid-like molecules are acetylated by Atf2 and deacetylated by Say1. Lack of deacetylation results in export of the acetylated compound. The proposed separation between sterols that have passed the cycle from those that have not is suggested to prevent a futile cycle that would consume acetyl-CoA and hence waste energy.

suggesting that this putative detoxification cycle is operating under both growth conditions (Supplementary Figure S6).

Atf1p and Atf2p have homology to another ER protein, Sli1p, which *N*-acetylates myriocin and confers resistance against this sphingolipid inhibitor. In this case, however, acetylation serves to inactivate the drug rather than to excrete it, because the acetylated form of ISP-1/myriocin fails to bind to the target enzyme (Momoi *et al*, 2004). In vertebrates, on the other hand, sulfonation and glucuronoidation of sterols and steroids is important for detoxification and excretion of these otherwise poorly soluble compounds (Tukey and Strassburg, 2000; Strott, 2002).

The observation that expression of the human homologue of *SAY1*, AADAC, in yeast rescues the sterol acetate accumulation phenotype of *say1*Δ-mutant cells indicates that the human aryl acetamide deacetylase acts on cholesterol acetate and thus has overlapping substrate specificity with Say1 *in vivo*, even though the enzyme has been identified as an *N*-deacetylase based on its *in vitro* activity against aryl acetamide (Probst *et al*, 1994). The mouse AADAC is expressed in liver, intestinal mucosa, the pancreas, and also the adrenal gland, and has been proposed to play a role in promoting the mobilization of lipids from internal stores and in the liver for assembling VLDL (Trickett *et al*, 2001). One of the orthologues of AADAC, AADACL1, has recently been identified as a detoxifying enzyme for organophosphorus nerve poisons, and null mutant mice are viable and phenotypically normal under standard conditions (Nomura *et al*, 2005).

The human genome has no identifiable homologue of the acetyltransferase Atf2. We thus examined whether pregnenolone is acetylated by human cells. Incubation of HepG2 hepatoma cells with radiolabeled pregnenolone, however, did not reveal any conversion to pregnenolone acetate or export of modified pregnenolone into the culture media, indicating that the substrate specificity of a putative lipid acetylase, if present in mammals, is different from that of yeast cells (data not shown).

In *Mycoplasma capricolum*, cholesterol acetate can replace cholesterol for growth (Lala *et al*, 1979). Our observation that cholesterol acetate is selectively secreted from yeast cells lacking Say1, however, indicates that cells have evolved mechanism that allow to distinguish cholesterol from cholesterol acetate even though both lipids can form membranes *in vitro* (Kwong *et al*, 1971).

While steroids were thought to passively diffuse through cellular membranes as postulated by the free hormone hypothesis (Mendel, 1989), there is now increasing evidence that these poorly water soluble compounds are transported by plasma carriers and then taken up by receptor-mediated endocytosis of loaded carriers similar to uptake of cholesterol by the LDL receptor (Hammes *et al*, 2005). Export of steroids from certain mammalian cells growing in culture, on the other hand, is temperature and energy dependent, and a saturable process that acts on selected steroids only (Gross *et al*, 1970). Our observation that export of cholesterol acetate requires ongoing vesicular transport from the ER, whereas export of pregnenolone acetate is independent of secretion indicates that yeast cells have at least two distinct mechanisms to export acetylated sterols and steroids. Further genetic and biochemical approaches are now required to define the mechanism that governs export of pregnenolone acetate from yeast cells in more detail.

In mammals, oxidation of cholesterol to either 7α- or 27-hydroxycholesterol, followed by further oxidation to bile acids comprises a major pathway of cholesterol catabolism (Russell, 2003). In this case, cholesterol is rendered more hydrophilic and ultimately water soluble to allow its excretion via the bile. In contrast, the acetylation cycle described here for yeast appears to follow a different strategy as acetylation renders the lipid even more hydrophobic. This opens the question how cholesterol acetate is rendered soluble, either in the ER lumen or after it has been secreted into the culture supernatant. One possibility might be that cholesterol acetate is selectively recognized and binds to a soluble protein, possibly already within the ER lumen, and is then also rendered soluble through this protein interaction after secretion. Further studies are now under way to identify such a putative cholesterol acetate-binding protein.

Materials and methods

Yeast strains and growth conditions

Yeast strains used in this study are listed in Supplementary Table SI. Strains bearing single deletions of non-essential genes were obtained from EUROSCARF. Strains were cultivated in YPD rich or minimal media. Media supplemented with sterols and fatty acids contained 0.05 mg/ml Tween 80 and 20 μg/ml ergosterol or cholesterol (Sigma Chemical Co., St Louis, MO). Alternatively, *hem1*Δ-mutant cells were supplemented with 10 μg/ml Δ-aminolevulinic acid (ALA). Site-directed mutagenesis was performed as previously described (Toulmay and Schneiter, 2006). Expression clones for AADAC and AADACL1 were obtained from the Deutsches Resourcenzentrum für Genomforschung (RZPD, Berlin-Charlottenburg, Germany).

Subcellular fractionation

Subcellular fractionation, detergent and salt extractions, and proteinase K treatment were performed essentially as previously described (Köffel *et al*, 2005).

Deglycosylation by endoglycosidase H was performed by incubating total cell extracts with 200 mU of EndoH (Roche-Diagnostics, Rotkreuz, Switzerland) at 37°C for 16 h. Proteins were precipitated with TCA and analyzed by western blot.

Fluorescence microscopy

Fluorescence microscopy of living cells was performed using a Zeiss Axioplan 2 (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam CCD camera and AxioVision 3.1 software. Indirect immunofluorescence was performed using mouse anti-myc and rabbit anti-Sec61 antibodies. Secondary antibodies used were FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit (both 1:200; Sigma) antibodies. Nuclei were visualized by DAPI (4,6-diamidino-2-phenylindole) staining.

Lipid labeling and analysis

Uptake of [^{14}C]cholesterol was performed essentially as described (Reiner *et al*, 2006). *hem1*Δ-mutant cells were cultured in cholesterol/Tween-containing media and labeled with 0.025 μCi/ml [^{14}C]cholesterol (American Radiolabeled Chemicals Inc., St Louis, MO) for 16 h at 24°C. Cells were diluted to OD 0.5 in fresh medium containing cold cholesterol and grown for 4 h. Cells were disrupted with glass beads in the presence of chloroform/methanol (1:1) and lipids were extracted. Lipids were separated by TLC on silica gel 60 plates (TLC; Merck, Darmstadt, Germany) with the solvent system petroleum ether/diethylether/acetic acid (70:30:2; per volume), and radiolabeled lipids were quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer (Berthold Technologies, Bad Wildbad, Germany). TLC plates were then exposed and visualized using a phosphorimager (Bio-Rad Laboratories, Hercules, CA). Lipids were deacylated by treatment with 0.1 M NaOH for 60 min at 30°C.

Newly synthesized ergosterol was labeled by incubating cells with 10 μCi/ml L-[methyl-^{3}H]methionine (85 Ci/mmol; American Radiolabeled Chemicals Inc.) for 16 h at 24°C.

To examine sterol export into the culture media, cells were labeled with [^{14}C]cholesterol (0.025 µCi/ml), for 16 h at 24°C. Cells were collected by centrifugation, washed twice with YPD, diluted to OD_{600} of ~1, and grown for the time indicated. Lipids were extracted from the cell pellet and the culture media. Samples were dried and analyzed by TLC.

To examine pregnenolone export, cells were labeled with 1 µCi/ml [^{3}H]pregnenolone (American Radiolabeled Chemicals Inc.) for 2 h at 24°C. Cells were collected by centrifugation and lipids were extracted from the cell pellet and the culture media and analyzed by TLC.

In vitro deacetylase assay

Microsomal membranes were incubated with 0.3 mg/ml *p*-nitrophenyl acetate or *p*-nitrophenyl palmitate in 100 mM potassium phosphate, pH 6.9, 50 mM Mg_2Cl_2 for 30 min at 30°C. The reaction was stopped by the addition of Triton X-100 to 2%; samples were placed on ice and cleared of precipitates by centrifugation. Conversion of the acylated *p*-nitrophenyl to *p*-nitrophenol was determined colorimetrically by reading absorbance at 410 nm (Gupta *et al*, 2002).

Acknowledgements

We thank O Aebischer for help with mass spectrometry, D Picard and V Sundaramurthy for comments on the manuscript, and the Swiss National Science Foundation (631-065925 and PP00A-110450) for financial support.

References

Adams CM, Reitz J, De Brabander JK, Feramisco JD, Li L, Brown MS, Goldstein JL (2004) Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. *J Biol Chem* **279:** 52772–52780

Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* **308:** 1466–1469

Arpigny JL, Jaeger KE (1999) Bacterial lipolytic enzymes: classification and properties. *Biochem J* **343:** 177–183

Barlowe C, Schekman R (1993) *SEC12* encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. *Nature* **365:** 347–349

Cauet G, Degryse E, Ledoux C, Spagnoli R, Achstetter T (1999) Pregnenolone esterification in *Saccharomyces cerevisiae*. A potential detoxification mechanism. *Eur J Biochem* **261:** 317–324

Chang TY, Chang CC, Ohgami N, Yamauchi Y (2006) Cholesterol sensing, trafficking, and esterification. *Annu Rev Cell Dev Biol* **22:** 129–157

Daum G, Lees ND, Bard M, Dickson R (1998) Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. *Yeast* **14:** 1471–1510

Derewenda ZS, Derewenda U (1991) Relationships among serine hydrolases: evidence for a common structural motif in triacylglyceride lipases and esterases. *Biochem Cell Biol* **69:** 842–851

Gao XD, Tachikawa H, Sato T, Jigami Y, Dean N (2005) Alg14 recruits Alg13 to the cytoplasmic face of the endoplasmic reticulum to form a novel bipartite UDP-*N*-acetylglucosamine transferase required for the second step of *N*-linked glycosylation. *J Biol Chem* **280:** 36254–36262

Gross SR, Aronow L, Pratt WB (1970) The outward transport of cortisol by mammalian cells *in vitro*. *J Cell Biol* **44:** 103–114

Gupta N, Rathi P, Gupta R (2002) Simplified para-nitrophenyl palmitate assay for lipases and esterases. *Anal Biochem* **311:** 98–99

Haines TH (2001) Do sterols reduce proton and sodium leaks through lipid bilayers? *Prog Lipid Res* **40:** 299–324

Hammes A, Andreassen TK, Spoelgen R, Raila J, Hubner N, Schulz H, Metzger J, Schweigert FJ, Luppa PB, Nykjaer A, Willnow TE (2005) Role of endocytosis in cellular uptake of sex steroids. *Cell* **122:** 751–762

Hemila H, Koivula TT, Palva I (1994) Hormone-sensitive lipase is closely related to several bacterial proteins, and distantly related to acetylcholinesterase and lipoprotein lipase: identification of a superfamily of esterases and lipases. *Biochim Biophys Acta* **1210:** 249–253

Köffel R, Tiwari R, Falquet L, Schneiter R (2005) The *Saccharomyces cerevisiae YLL012/YEH1*, *YLR020/YEH2*, and *TGL1* genes encode a novel family of membrane-anchored lipases that are required for steryl ester hydrolysis. *Mol Cell Biol* **25:** 1655–1668

Kolaczkowski M, van der Rest M, Cybularz-Kolaczkowska A, Soumillion JP, Konings WN, Goffeau A (1996) Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p. *J Biol Chem* **271:** 31543–31548

Kralli A, Bohen SP, Yamamoto KR (1995) LEM1, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones. *Proc Natl Acad Sci USA* **92:** 4701–4705

Kwong CN, Heikkila RE, Cornwell DG (1971) Properties of cholesteryl esters in pure and mixed monolayers. *J Lipid Res* **12:** 31–35

Lala AK, Buttke TM, Bloch K (1979) On the role of the sterol hydroxyl group in membranes. *J Biol Chem* **254:** 10582–10585

Lewis TA, Taylor FR, Parks LW (1985) Involvement of heme biosynthesis in control of sterol uptake by *Saccharomyces cerevisiae*. *J Bacteriol* **163:** 199–207

Mahe Y, Lemoine Y, Kuchler K (1996) The ATP binding cassette transporters Pdr5 and Snq5 of *Saccharomyces cerevisiae* can mediate transport of steroids *in vivo*. *J Biol Chem* **271:** 25167–25172

Mason AB, Dufour JP (2000) Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* **16:** 1287–1298

Mendel CM (1989) The free hormone hypothesis: a physiologically based mathematical model. *Endocr Rev* **10:** 232–274

Momoi M, Tanoue D, Sun Y, Takematsu H, Suzuki Y, Suzuki M, Suzuki A, Fujita T, Kozutsumi Y (2004) *SLI1* (*YGR212W*) is a major gene conferring resistance to the sphingolipid biosynthesis inhibitor ISP-1, and encodes an ISP-1 *N*-acetyltransferase in yeast. *Biochem J* **381:** 321–328

Nakamura K, Niimi M, Niimi K, Holmes AR, Yates JE, Decottignies A, Monk BC, Goffeau A, Cannon RD (2001) Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters. *Antimicrob Agents Chemother* **45:** 3366–3374

Nilsson I, von Heijne G (2000) Glycosylation efficiency of Asn–Xaa–Thr sequons depends both on the distance from the C terminus and on the presence of a downstream transmembrane segment. *J Biol Chem* **275:** 17338–17343

Nomura DK, Durkin KA, Chiang KP, Quistad GB, Cravatt BF, Casida JE (2006) Serine hydrolase KIAA1363: toxicological and structural features with emphasis on organophosphate interactions. *Chem Res Toxicol* **19:** 1142–1150

Nomura DK, Leung D, Chiang KP, Quistad GB, Cravatt BF, Casida JE (2005) A brain detoxifying enzyme for organophosphorus nerve poisons. *Proc Natl Acad Sci USA* **102:** 6195–6200

Probst MR, Beer M, Beer D, Jeno P, Meyer UA, Gasser R (1994) Human liver arylacetamide deacetylase. Molecular cloning of a novel esterase involved in the metabolic activation of arylamine carcinogens with high sequence similarity to hormone-sensitive lipase. *J Biol Chem* **269:** 21650–21656

Reiner S, Micolod D, Zellnig G, Schneiter R (2006) A genomewide screen reveals a role of mitochondria in anaerobic uptake of sterols in yeast. *Mol Biol Cell* **17:** 90–103

Rodriguez RJ, Arunachalam TA, Parks LW, Caspi E (1983) growth of a sterol auxotroph derived from *Saccharomyces cerevisiae* on chemically synthesized derivatives of cholesterol possessing side-chain modifications. *Lipids* **18:** 772–775

Russell DW (2000) Oxysterol biosynthetic enzymes. *Biochim Biophys Acta* **1529:** 126–135

Russell DW (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Annu Rev Biochem* **72:** 137–174

Strott CA (2002) Sulfonation and molecular action. *Endocr Rev* **23:** 703–732

Toulmay A, Schneiter R (2006) A two-step method for the introduction of single or multiple defined point mutations into the genome of *Saccharomyces cerevisiae*. *Yeast* **23:** 825–831

Trickett JI, Patel DD, Knight BL, Saggerson ED, Gibbons GF, Pease RJ (2001) Characterization of the rodent genes for arylacetamide deacetylase, a putative microsomal lipase, and evidence for transcriptional regulation. *J Biol Chem* **276:** 39522–39532

Tukey RH, Strassburg CP (2000) Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* **40:** 581–616

Umebayashi K, Nakano A (2003) Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane. *J Cell Biol* **161:** 1117–1131

Verstrepen KJ, Van Laere SD, Vanderhaegen BM, Derdelinckx G, Dufour JP, Pretorius IS, Winderickx J, Thevelein JM, Delvaux FR (2003) Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. *Appl Environ Microbiol* **69:** 5228–5237

Vico P, Cauet G, Rose K, Lathe R, Degryse E (2002) Dehydroepiandrosterone (DHEA) metabolism in *Saccharomyces cerevisiae* expressing mammalian steroid hydroxylase CYP7B: Ayr1p and Fox2p display 17beta-hydroxysteroid dehydrogenase activity. *Yeast* **19:** 873–886

Yang H, Bard M, Bruner DA, Gleeson A, Deckelbaum RJ, Aljinovic G, Pohl TM, Rothstein R, Sturley SL (1996) Sterol esterification in yeast: a two-gene process. *Science* **272:** 1353–1356

Yu C, Kennedy NJ, Chang CC, Rothblatt JA (1996) Molecular cloning and characterization of two isoforms of *Saccharomyces cerevisiae* acyl-CoA:sterol acyltransferase. *J Biol Chem* **271:** 24157–24163